

NOVEL HUMAN MITOCHONDRIAL PROTEINS AND  
POLYNUCLEOTIDES ENCODING THE SAME

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The present application claims benefit of U.S. provisional  
application serial number <sup>60</sup>~~06~~/207,933, filed May 30, 2000, the  
5 disclosure of which is herein incorporated by reference in its  
entirety.

1. INTRODUCTION

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The present invention relates to the discovery,  
identification, and characterization of a novel human  
10 polynucleotide sequence and the novel polypeptide encoded  
thereby. The invention encompasses the described  
polynucleotides, host cell expression systems, the encoded  
protein or polypeptide, and fusion proteins and peptides  
derived therefrom, antibodies to the encoded protein or  
15 peptides, and genetically engineered animals that lack the  
disclosed polynucleotides, over express the disclosed  
polynucleotides, as well as antagonists and agonists of the  
protein, along with other compounds that modulate the  
expression or activity of the protein encoded by the disclosed  
20 polynucleotides that can be used for diagnosis, drug screening,  
clinical trial monitoring, the treatment of diseases and  
disorders, and cosmetic or nutraceutical applications.

2. BACKGROUND OF THE INVENTION

Many proteins present in the mitochondria are encoded  
25 within the nucleus. In the mitochondria, such nuclear encoded  
proteins are involved in, or regulate, the gradient that drives  
energy production in the cell/body. Accordingly, such proteins  
effectively modulate the efficiency of energy production in the  
body, and hence body metabolism. Given the role of such  
30 proteins in the body, they are thought to be important targets  
for the study of thermogenesis, obesity, cachexia, and other

metabolically related physiological functions, diseases, and disorders.

### 3. SUMMARY OF THE INVENTION

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The present invention relates to the discovery,  
5 identification, and characterization of nucleotides that encode novel human mitochondrial proteins that are encoded in the nucleus, and the corresponding amino acid sequences encoded by the disclosed sequences. The novel human proteins (NHPs) described for the first time herein share structural motifs  
10 typical of mammalian mitochondrial solute carriers, RNA splicing proteins, uncoupling proteins, and mitochondrial carrier proteins. The novel human nucleic acid sequence described herein encode proteins of 364, 193, 230, 265, 94, and 131 amino acids in length (see SEQ ID NOS: 2, 4, 6, 8, 10, and  
15 12).

An additional aspect of the present invention includes knockout cells and animals having genetically engineered mutations in gene encoding the presently described NHPs.

The invention encompasses the nucleotides presented in the  
20 Sequence Listing, host cells expressing such nucleotides, and the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHPs and the NHP products; (b) nucleotides that encode one or more portions of a  
25 NHP that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a  
30 part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d)

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nucleotides that encode chimeric fusion proteins containing all  
or a portion of a coding region of a NHP, or one of its domains  
(e.g., a transmembrane domain, accessory protein/self-  
association domain, etc.) fused to another peptide or  
5 polypeptide.

The invention also encompasses agonists and antagonists of  
the described NHPs, including small molecules, large molecules,  
mutant NHPs, or portions thereof, that compete with native NHP,  
peptides, and antibodies, as well as nucleotide sequences that  
10 can be used to inhibit the expression of the described NHPs  
(e.g., antisense and ribozyme molecules, and gene or regulatory  
sequence replacement constructs) or to enhance the expression  
of the described NHP polynucleotides (e.g., expression  
constructs that place the described polynucleotide under the  
15 control of a strong promoter system), and transgenic animals  
that express a NHP transgene, or "knock-outs" (which can be  
conditional) that do not express a functional NHP. Knock-out  
mice can be produced in several ways, one of which involves the  
use of mouse embryonic stem cells ("ES cells") lines that  
20 contain gene trap mutations in a murine homolog of at least one  
of the described NHPs. When the unique NHP sequences described  
in SEQ ID NOS:1-12 are "knocked-out" they provide a method of  
identifying phenotypic expression of the particular gene as  
well as a method of assigning function to previously unknown  
25 genes. Additionally, the unique NHP sequences described in SEQ  
ID NOS:1-12 are useful for the identification of coding  
sequence and the mapping a unique gene to a particular  
chromosome.

Further, the present invention also relates to processes  
30 for identifying compounds that modulate, i.e., act as agonists  
or antagonists, of NHP expression and/or NHP activity that  
utilize purified preparations of the described NHPs and/or NHP  
product, or cells expressing the same. Such compounds can be

used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

5           4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides polynucleotides encoding the described NHPs and the amino acid sequence encoded thereby.

          5. DETAILED DESCRIPTION OF THE INVENTION

10           The NHPs, described for the first time herein, are novel proteins that are expressed, *inter alia*, in gene trapped human cells, human fetal brain, pituitary, trachea, lung, salivary gland, and fetal kidney cells. The NHPs exert biological effect by directly or indirectly regulating the efficiency of  
15 energy generation in the body with the result being that excess resources are converted to heat or other wise stored as fat, etc. Regulating the function of a NHP product will effect NHP-mediated processes with a resulting effects on fat production and usage, superoxide generation and regulation, and all  
20 biological properties and functions that are tied to fatty acid metabolism. Because of these important roles, similar proteins, e.g., UCPs, have been the focus of intense scientific scrutiny (see PCT Application No. PCT/EP98/02645, U.S. Patent Nos. 5,853,975, 5,741,666 and 5,702,902 all of which are herein  
25 incorporated by reference in their entirety).

The present invention encompasses the use of the described NHP nucleotides, NHPs and NHP peptides, as well as antibodies, preferably humanized monoclonal antibodies, or binding fragments, domains, or fusion proteins thereof, or  
30 antiidiotypic variants derived therefrom, that bind a NHP (which can, for example, also act as NHP agonists or antagonists), other antagonists that inhibit binding activity or expression, or agonists that activate NHP receptor activity

or increase NHP expression, in the diagnosis and/or treatment of disease.

In particular, the invention described in the subsections below encompasses NHP polypeptides or peptides corresponding to functional domains of NHPs, mutated, truncated or deleted NHPs (e.g., NHPs missing one or more functional domains or portions thereof), NHP fusion proteins (e.g., where a NHP or a functional domain of a NHP is fused to an unrelated protein or peptide such as an immunoglobulin constant region, i.e., IgFc), nucleotide sequences encoding such products, and host cell expression systems that can produce such NHP products.

The invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.). The invention also relates to host cells and animals genetically engineered to express a NHP (or mutant variants thereof) or to inhibit or "knock-out" expression of an animal homolog or ortholog of a NHP gene.

The NHPs or NHP peptides and fusion proteins derived therefrom, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of biological disorders (high blood pressure, obesity, etc.) and disease. The NHP products or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput

screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in  
5 that such systems allow not only for the identification of compounds that bind to endogenous NHP, but can also identify compounds that facilitate or inhibit NHP-mediated uncoupling or solute flow in the mitochondria.

Of particular interest are genetically engineered  
10 nucleotide constructs, or expression vectors, that encode NHP products and derivatives (NHP peptides, fusions, etc). Nucleotide constructs encoding such NHP products and derivatives can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered  
15 cells function as "bioreactors" in the body delivering a continuous supply of a NHP product, NHP peptide, or NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the  
20 modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Therapeutic gene delivery of the described NHP nucleotides can be effected by a variety of methods. For example, methods  
25 of retroviral human gene therapy are described in, *inter alia*, U.S. Patents Nos. 5,399,346 and 5,858,740; adenoviral vectors for gene therapy/delivery are described in U.S. Patent No. 5,824,544; adeno-associated viral vectors are described in U.S. Patents Nos. 5,843,742, 5,780,280, and 5,846,528; herpes virus  
30 vectors are described in U.S. Patent No. 5,830,727, and other vectors and methods of nonvirally (e.g., polynucleotides that are not encapsulated by viral capsid protein, "naked" DNA, or DNA formulated in lipid or chemical complexes) introducing foreign genetic material of recombinant origin into a host

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mammalian, and preferably human, cell are described in U.S. Patents Nos. 5,827,703 and 5,840,710 all of which are herein incorporated by reference in their entirety. When the above methods are applied to selectively express, or inhibit the expression of, NHP in tumor/diseased cells, the described methods and compositions can be used as chemotherapeutic agents for the treatment of cancer and other diseases and disorders.

Various aspects of the invention are described in greater detail in the subsections below.

#### 5.1. THE NHP SEQUENCES

The cDNA sequences and deduced amino acid sequence of the described NHPs are presented in the Sequence Listing. The NHP sequences were obtained from a human pituitary gland cDNA library (Edge Biosystems, Gaithersburg, MD) and RT-PCR products generated using fetal brain, brain, pituitary gland, and testis mRNA using probes and/or primers generated from gene trapped sequence tags and a human homolog of the described NHP.

The NHPs of the present invention includes: (a) the human DNA sequences presented in the Sequence Listing and additionally contemplates any nucleotide sequence encoding a contiguous and functional NHP open reading frame (ORF) that hybridizes to a complement of the DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g.,

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washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet still encode a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species, and mutant NHPs whether  
5 naturally occurring or engineered. The invention also includes degenerate variants of the disclosed sequences.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP nucleotide sequences.  
10 Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are particularly about 16 to about 100 bases long, about 20 to about 80, or about 34 to about 45 bases  
15 long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the present Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and  
20 prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the  
25 described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-12 can be used as a  
30 hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics, polymers, metal or metallized substrates, crystalline or polycrystalline substrates, etc.). Of particular note are spatially addressable arrays (*i.e.*, gene chips, microtiter plates, etc.)



of oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-12, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, *inter alia*, U.S. Patent Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-12 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-12.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can

begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

5           Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ  
10 ID NOS:1-12 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

          Probes consisting of sequences first disclosed in SEQ ID  
15 NOS:1-12 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct  
20 from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

          As an example of utility, the sequences first disclosed in SEQ ID NOS:1-12 can be utilized in microarrays or other assay  
25 formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences first disclosed in SEQ ID NOS:1-12 *in silico* and by comparing previously collected genetic databases and the disclosed  
30 sequences using computer software known to those in the art.

          Thus the sequences first disclosed in SEQ ID NOS:1-12 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

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Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific oligonucleotide sequence(s) first disclosed in the SEQ ID NOS: 1-12. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (e.g., the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, MI, etc.), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for NHP gene regulation.

Additionally, the antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the

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strands run parallel to each other (Gautier *et al.*, 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue *et al.*, 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 5 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, 10 phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

15 Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory 20 Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library 25 using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms, determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to 30 the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice

acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog may be isolated from nucleic acid of the organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP product disclosed herein. The template for the reaction can be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue, such as choroid plexus, known or suspected to express a NHP gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene, such as, for example, brain tissue). A reverse transcription (RT) reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et al., 1989, *supra*.

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A cDNA of a mutant NHP gene may be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of the normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry the mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. The normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing the mutant NHP gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies

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raised against the normal NHP product, as described, below, in Section 5.3. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, NY.)

5        Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result  
10 of a missense or a frameshift mutation), a polyclonal set of antibodies to NHP are likely to cross-react with the mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

15        The invention also encompasses nucleotide sequences that encode mutant NHPs, peptide fragments of NHPs, truncated NHPs, and NHP fusion proteins. These include, but are not limited to nucleotide sequences encoding the mutant NHPs described in section 5.2 *infra*; polypeptides or peptides corresponding to  
20 one or more domains of NHP or portions of these domains; truncated NHPs in which one or more of the domains is deleted, or a truncated nonfunctional NHPs. Nucleotides encoding fusion proteins may include, but are not limited to, full length NHP sequences, truncated NHPs, or nucleotides encoding peptide  
25 fragments of a NHP fused to an unrelated protein or peptide, such as for example, a NHP domain fused to an Ig Fc domain which increases the stability and half life of the resulting fusion protein (e.g., NHP-Ig) in the bloodstream; or an enzyme such as a fluorescent protein or a luminescent protein which  
30 can be used as a marker.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (*i.e.*, antisense); (b) DNA expression vectors that



contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast  $\alpha$ -mating factors.

An additional application of the described novel human polynucleotide sequences is their use in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Patents Nos. 5,830,721 and 5,837,458 which are herein incorporated by reference in their entirety.

NHP gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate NHP transgenic animals.

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Any technique known in the art may be used to introduce a NHP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the NHP transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.*, mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that a NHP transgene be integrated into the chromosomal site of the endogenous NHP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NHP gene are designed for the purpose of integrating, via homologous recombination with

chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NHP gene (i.e., "knockout" animals).

5 The transgene can also be selectively introduced into a particular cell type, thus inactivating the endogenous NHP gene in only that cell type, by following, for example, the teaching of Gu et al., 1994, Science, 265:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will  
10 be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NHP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal  
15 tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, *in situ*  
20 hybridization analysis, and RT-PCR. Samples of NHP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the NHP transgene product.

## 5.2. NHP POLYPEPTIDES AND PEPTIDES DERIVED THEREFROM

25 NHPs, NHP polypeptides, NHP peptide fragments, mutated, truncated, or deleted forms of NHP, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include but are not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene  
30 products related to a NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease.

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The Sequence Listing discloses the amino acid sequences encoded by the described NHP gene. The NHP sequence has an initiator methionine in a DNA sequence context consistent with a translation initiation site (Kozak sequence).

5       The NHP sequences of the invention include the nucleotide and amino acid sequences presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP  
10 nucleotide sequences described in Section 5.1, above, are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and,  
15 accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken  
20 together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell *et al.* eds., Scientific American Books, New York, NY, herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid  
25 sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHP encoded by the nucleotide sequences described in Section 5.1, as judged by any of a number of criteria, including, but not limited to, the ability  
30 to partition into the mitochondria, or other cellular membrane structure, and effect uncoupling activity, change in cellular metabolism (e.g., ion flux, tyrosine phosphorylation, etc.), or change in phenotype when the NHP equivalent is expressed at similar levels, or mutated, in an appropriate cell type (such

as the amelioration, prevention or delay of a biochemical, biophysical, or overt phenotype). Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by  
5 site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Patent No. 5,837,458). The invention also includes degenerate nucleic acid variants and splice variant of the disclosed NHP polynucleotide sequence.

Additionally contemplated are polynucleotides encoding NHP  
10 ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG  
15 sequence analysis package using standard default settings).

Functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, in Section 5.1, but which result in  
20 a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids  
25 include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively  
30 charged (acidic) amino acids include aspartic acid and glutamic acid.

While random mutations can be made to NHP encoding DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant NHPs tested for

activity, site-directed mutations of the NHP coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant NHPs with increased function, e.g., higher receptor binding

5 affinity, decreased function, and/or increased physiological half-life, and increased or decreased activity. One starting point for such analysis is by aligning the disclosed human sequences with corresponding gene/protein sequences from, for example, other mammals in order to identify amino acid sequence

10 motifs that are conserved between different species. Non-conservative changes can be engineered at variable positions to alter function, signal transduction capability, or both. Alternatively, where alteration of function is desired, deletion or non-conservative alterations of the conserved

15 regions (i.e., identical amino acids) can be engineered. For example, deletion or non-conservative alterations (substitutions or insertions) of the various conserved transmembrane domains.

Other mutations to a NHP coding sequence can be made to

20 generate NHPs that are better suited for expression, scale up, etc. in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid in order to eliminate disulfide bridges; N-linked glycosylation sites can be altered or eliminated to achieve, for example,

25 expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions of any one or more of the glycosylation

30 recognition sequences which occur in an ECD (N-X-S or N-X-T), and/or an amino acid deletion at the second position of any one or more such recognition sequences in an ECD will prevent glycosylation of the NHP at the modified tripeptide sequence. (See, e.g., Miyajima et al., 1986, EMBO J. 5(6):1193-1197).

Peptides corresponding to one or more domains of a NHP, truncated or deleted NHPs, as well as fusion proteins in which full length NHP, a NHP peptide, or truncated NHP is fused to an unrelated protein, are also within the scope of the invention and can be designed on the basis of the presently disclosed NHP polynucleotide sequences and NHP amino acid sequences. Such fusion proteins include, but are not limited to, IgFc fusions which stabilize the NHP protein, or NHP peptides, and prolong half-life *in vivo*; or fusions to any amino acid sequence that allows the fusion protein to be anchored to the cell membrane; or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the NHPs and peptides can be chemically synthesized (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y.), large polypeptides derived from full length NHP can be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acids containing NHP gene sequences and/or coding sequences. Such methods can be used to construct expression vectors containing the NHP gene nucleotide sequences described in Section 5.1 and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook *et al.*, 1989, *supra*, and Ausubel *et al.*, 1989, *supra*. Alternatively, RNA corresponding to all or a portion of a transcript encoded by the NHP gene sequence may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the NHP gene nucleotide sequences of the invention. Where the NHP peptide or polypeptide is a soluble derivative (e.g., NHP peptides corresponding to an ECD; truncated or deleted NHP in which a TM and/or CD are deleted, etc.) the peptide or polypeptide can be recovered from the culture, i.e., from the host cell in cases where the NHP peptide or polypeptide is not secreted, and from the culture media in cases where the NHP peptide or polypeptide is secreted by the cells. However, such expression systems also encompass engineered host cells that express NHP, or a functional equivalent thereof, *in situ*, i.e., anchored in the cell membrane. Purification or enrichment of NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that can be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or



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mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The NHP gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for

example the polyhedrin promoter). Successful insertion of the NHP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP gene nucleotide sequence of interest may be ligated to an adenovirus transcription and/or translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing NHP in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of NHP transcripts. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP sequence or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural

and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, Methods in Enzymol. 153:516-544).

5 In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function  
10 of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.  
15 To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in  
20 particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the presently described NHP may be engineered. Rather than using expression vectors which contain  
25 viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells  
30 may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their

chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express NHP. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk<sup>-</sup>, hgp<sup>r</sup>t<sup>-</sup> or ap<sup>r</sup>t<sup>-</sup> cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with

recombinant vaccinia virus are loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

NHP products can also be expressed in transgenic animals. 5 Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate NHP transgenic animals.

Any technique known in the art may be used to introduce a 10 NHP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. 15 Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723); etc. For a review of such techniques, 20 see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry a NHP transgene in all of their cells, as well as animals 25 which carry the transgene in some, but not all their cells, i.e., mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into 30 and activated in a particular cell type by following, for example, the teaching of Lasko *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the

particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the NHP transgene be integrated into the chromosomal site of the endogenous NHP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NHP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NHP gene (*i.e.*, "knockout" animals).

The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous NHP gene in only that cell type, by following, for example, the teaching of Gu *et al.*, 1994, *Science*, 265:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NHP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of NHP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the NHP transgene product.

Also encompassed by the present invention are fusion proteins that direct the NHP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHPs to antibody molecules or their Fab

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fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHP would also transport the NHP to the desired location within the cell. Alternatively targeting of NHP or its nucleic acid  
5 sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in "Liposomes: A Practical Approach", New, RRC ed., Oxford University Press, New York and in U.S. Patents Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective  
10 disclosures which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHP to the target site or desired organ. This goal may be achieved by coupling of the NHP to a cytokine or other ligand  
15 that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. applications Ser. No. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes if  
20 needed and can optionally be engineered to include nuclear localization sequences when desired.

### 5.3. ANTIBODIES TO NHPS

Antibodies that specifically recognize one or more  
25 epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments,  
30 F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of a NHP in a biological sample and may,

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therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of a NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described, 5 below, in Section 5.5, for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the 10 patient. Such antibodies may additionally be used as a method for inhibiting abnormally high NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals can 15 be immunized by injection with the NHP, a NHP peptide (e.g., one corresponding to a functional domain of a NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutants of the NHP. Such host animals may include but are not limited to 20 rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active 25 substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole 30 limpet hemocyanin, tetanus toxoid, diptheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.



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Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, 5 but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma 10 technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. 15 Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604- 20 608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived 25 from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Patents Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their 30 entirety. Also encompassed by the present invention is the use of fully humanized monoclonal antibodies as described in US Patent No. 6,150,584 and respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 341:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies that bind to a NHP domain and competitively inhibit the binding of NHP to its cognate ligand, chaperonin, or accessory molecule(s) can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving the NHP signaling pathway.

#### 5.4. DIAGNOSIS OF ABNORMALITIES RELATED TO A NHP

5 A variety of methods can be employed for the diagnostic and prognostic evaluation of disorders related to NHP function, and for the identification of subjects having a predisposition to such disorders.

10 Such methods may, for example, utilize reagents such as the NHP nucleotide sequences described in Section 5.1, and NHP antibodies, as described, in Section 5.3. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of NHP gene mutations, or the detection of either over- or under-expression of NHP mRNA relative to a given phenotype; (2) the detection of either an over- or an under-abundance of NHP gene product relative to a given phenotype; and (3) the detection of perturbations or abnormalities in any  
15 metabolic, physiologic, catabolic, or biochemical pathway mediated by a NHP.

20 The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific NHP nucleotide sequence or NHP antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting, for example, body weight disorder abnormalities.

25 For the detection of NHP mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of NHP gene expression or NHP gene products, any cell type or tissue in which the NHP gene is expressed, such as, for example, kidney cells, may be utilized.

Nucleic acid-based detection techniques and peptide detection techniques are described below.

#### 5.4.1. DETECTION THE OF NHP GENE AND TRANSCRIPTS

Mutations within a NHP gene can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving NHP gene structure, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

Such diagnostic methods for the detection of NHP gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described in Section 5.1, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the NHP gene. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:NHP molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the

type described in Section 5.1 are easily removed. Detection of the remaining, annealed, labeled NHP nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The NHP gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal NHP gene sequence in order to determine whether a NHP gene mutation is present.

Alternative diagnostic methods for the detection of NHP gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of a NHP gene in order to determine whether a NHP gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals carrying NHP gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

Additionally, improved methods for analyzing DNA polymorphisms which can be utilized for the identification of NHP gene mutations have been described which capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217, which is incorporated herein by reference in its entirety) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> short tandem repeats. The average separation of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> blocks is estimated to be 30,000-60,000 bp. Markers which are

so closely spaced exhibit a high frequency co-inheritance, and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the NHP gene, and the diagnosis of diseases and disorders related to NHP mutations.

Also, Caskey *et al.* (U.S. Pat. No. 5,364,759, which is incorporated herein by reference in its entirety) describe a DNA profiling assay for detecting short tri and tetra nucleotide repeat sequences. The process includes extracting the DNA of interest, such as the NHP gene, amplifying the extracted DNA, and labeling the repeat sequences to form a genotypic map of the individual's DNA.

The level of NHP gene expression can also be assayed by detecting and measuring NHP transcription. For example, RNA from a cell type or tissue known, or suspected to express the NHP gene, such as kidney, may be isolated and tested utilizing hybridization or PCR techniques such as those described above. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the NHP gene. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the NHP gene, including activation or inactivation of NHP gene expression.

In one embodiment of such a detection scheme, cDNAs are synthesized from the RNAs of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the NHP nucleic acid reagents

described in Section 5.1. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining, by utilizing any other suitable nucleic acid staining method, or by sequencing.

10        Additionally, it is possible to perform such NHP gene expression assays "*in situ*", *i.e.*, directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described in  
15        Section 5.1 may be used as probes and/or primers for such *in situ* procedures (See, for example, Nuovo, G.J., 1992, "PCR In Situ Hybridization: Protocols And Applications", Raven Press, NY).

          Alternatively, if a sufficient quantity of the appropriate  
20        cells can be obtained, standard Northern analysis can be performed to determine the level of mRNA expression of the NHP gene.

#### 5.4.2. DETECTION OF NHP PRODUCTS

25        Antibodies directed against wild type or mutant NHPs, or conserved variants or peptide fragments thereof, as discussed above in Section 5.3, may also be used as diagnostics and prognostics, as described herein. Such diagnostic methods, may be used to detect abnormalities in the level of NHP gene  
30        expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of the NHP (besides mitochondria), and may be performed *in vivo* or *in vitro*, such as, for example, on biopsy tissue.

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For example, antibodies directed to one or more epitopes of NHP can be used *in vivo* to detect the pattern and level of expression of NHP in the body. Such antibodies can be labeled, e.g., with a radio-opaque or other appropriate compound and injected into a subject in order to visualize binding to the NHP expressed in the body using methods such as X-rays, CAT-scans, or MRI. Labeled antibody fragments, e.g., the Fab or single chain antibody comprising the smallest portion of the antigen binding region, are preferred for this purpose to promote crossing the blood-brain barrier and permit labeling of NHP expressed in the brain.

Additionally, any NHP fusion protein or NHP conjugated protein whose presence can be detected, can be administered. For example, NHP fusion or conjugated proteins labeled with a radio-opaque or other appropriate compound can be administered and visualized *in vivo*, as discussed, above for labeled antibodies. Further such NHP fusion proteins (such as AP-NHP or NHP-AP) can be utilized for *in vitro* diagnostic procedures.

Alternatively, immunoassays or fusion protein detection assays, as described above, can be utilized on biopsy and autopsy samples *in vitro* to permit assessment of the expression pattern of the NHP. Such assays are not confined to the use of antibodies that define a NHP domain, but can include the use of antibodies directed to epitopes of any domain of a NHP. The use of each or all of these labeled antibodies will yield useful information regarding translation and intracellular transport of the NHP to the cell surface and can identify defects in processing.

The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the NHP gene, such as, for example, epithelial cells, kidney cells, adipose tissue, brain cells, etc. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988,



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"Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the NHP gene.

For example, antibodies, or fragments of antibodies, such as those described, above, in Section 5.3, useful in the present invention may be used to quantitatively or qualitatively detect the presence of NHP, or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below, this Section) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred if such NHP products can be found, at least transiently, on the cell surface.

The antibodies (or fragments thereof) or NHP fusion or conjugated proteins useful in the present invention may, additionally, be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for *in situ* detection of NHP gene products or conserved variants or peptide fragments thereof, or to assay NHP binding (in the case of labeled NHP-fusion protein).

*In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody or fusion protein of the present invention.

The antibody (or fragment) or fusion protein is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the NHP product, or conserved variants or peptide fragments, or NHP binding, but

also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to  
5 achieve such *in situ* detection.

Immunoassays and non-immunoassays for NHP, or conserved variants or peptide fragments thereof, will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which  
10 have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying NHP products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art. Alternatively, the labeled antibody can  
15 be directed against an antigenic tag that has been directly or indirectly attached to a NHP.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of  
20 immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled NHP antibody or NHP ligand/accessory molecule fusion protein. The solid phase support may then be washed with the buffer a second time to  
25 remove unbound antibody or fusion protein. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-  
30 known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present

invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, 5 or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, 10 or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of NHP antibody or NHP ligand fusion protein may be determined according to well known methods. Those skilled in the art will be able to determine 15 operative and optimal assay conditions for each determination by employing routine experimentation.

With respect to antibodies, one of the ways in which the NHP antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, 20 A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, A. et al., 1978, J. Clin. Pathol. 31:507-520; Butler, J.E., 1981, Meth. Enzymol. 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC 25 Press, Boca Raton, FL,; Ishikawa, E. et al., (eds.), 1981, Enzyme Immunoassay, Kaku Shoin, Tokyo). The enzyme that is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, 30 by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase,

triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The  
5 detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

10 Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect NHP through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays,  
15 Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

20 It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein  
25 isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the  
30 antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the

chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, 5 theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which 10 a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

15

#### 5.5. SCREENING ASSAYS FOR COMPOUNDS THATMODULATE NHP EXPRESSION OR ACTIVITY

The following assays are designed to identify compounds that interact with (e.g., bind to) NHP, compounds that 20 interfere with the interaction of NHP with any ligand or accessory molecules, and to compounds that modulate the activity of NHP gene expression (i.e., modulate the level of NHP gene expression) or modulate the levels of NHP in the body. Assays may additionally be utilized which identify compounds 25 that bind to NHP gene regulatory sequences (e.g., promoter sequences) and, consequently, may modulate NHP gene expression. See e.g., Platt, K.A., 1994, J. Biol. Chem. 269:28558-28562, which is incorporated herein by reference in its entirety.

The compounds which may be screened in accordance with the 30 invention include but are not limited to peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that bind to NHP and either mimic the activity of the natural product (i.e., agonists) or inhibit the activity of the natural ligand/accessory molecule (i.e., antagonists);

as well as peptides, antibodies or fragments thereof, and other organic compounds that mimic the NHP (or a portion thereof) and bind to and "inactivate" or "neutralize" the NHP ligand/accessory protein.

5        Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86), and combinatorial chemistry-derived  
10 molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778), antibodies (including, but not limited to, polyclonal,  
15 monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Other compounds that can be screened in accordance with  
20 the invention include but are not limited to small organic molecules that are able to cross the blood-brain barrier, gain entry into an appropriate cell (e.g., in the choroid plexus, pituitary, the hypothalamus, etc.) and affect the expression of a NHP gene or some other gene involved in a NHP mediated  
25 pathway (e.g., by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect or substitute for the activity of the NHP or the activity of some other intracellular factor involved in a NHP-mediated catabolic, or metabolic pathway.

30        Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate NHP expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites

might typically be ligand binding sites. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of  
5 complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

Next, the three dimensional geometric structure of the  
10 active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be  
15 used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is  
20 determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on  
25 computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry.  
30 The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site (or binding site), either experimentally, by modeling, or

by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined  
5 active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential NHP modulating compounds.

Alternatively, these methods can be used to identify  
10 improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered  
15 structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved  
20 specificity or activity.

Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites (or binding sites) of NHP, and related transduction and transcription factors will be apparent to  
25 those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling  
30 and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al.,



1988, Acta Pharmaceutical Fennica 97:159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, 1989, Annu. Rev. Pharmacol. Toxicol. 29:111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc. R. Soc. Lond. 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989, J. Am. Chem. Soc. 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Cell-based systems can also be used to identify compounds that bind (or mimic) NHP as well as assess the altered activity associated with such binding in living cells. One tool of particular interest for such assays is green fluorescent protein which is described, *inter alia*, in U.S. Patent No. 5,625,048, herein incorporated by reference. Cells that may be used in such cellular assays include, but are not limited to, leukocytes, or cell lines derived from leukocytes, lymphocytes, stem cells, including embryonic stem cells, and the like. In addition, expression host cells (e.g., B95 cells, COS cells, CHO cells, OMK cells, fibroblasts, Sf9 cells) genetically engineered to express a functional NHP of interest and to

respond to activation by the test, or natural, ligand, as measured by a chemical or phenotypic change, or induction of another host cell gene, can be used as an end point in the assay.

- 5       Compounds identified via assays such as those described herein may be useful, for example, in elucidating the biological function of NHP. Such compounds can be administered to a patient at therapeutically effective doses to treat any of a variety of physiological or mental disorders. A
- 10   therapeutically effective dose refers to that amount of the compound sufficient to result in any amelioration, impediment, prevention, or alteration of any biological symptom.

- Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell
- 15   cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>.
- 20   Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby,
- 25   reduce side effects.

- The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with
- 30   little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be

formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture.

- 5 Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner  
10 using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral,  
15 intracranial, intrathecal, or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize  
20 starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate).  
25 The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations  
30 may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or

fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

5       Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use  
10 according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable  
15 gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

20       The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions  
25 may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-  
30 free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

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In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

#### 5.5.1. *IN VITRO* SCREENING ASSAYS FOR COMPOUNDS THAT BIND TO A NHP

*In vitro* systems may be designed to identify compounds capable of interacting with (e.g., binding to) or mimicking NHP. The compounds identified can be useful, for example, in modulating the activity of wild type and/or mutant NHP; can be useful in elaborating the biological function of NHP; can be utilized in screens for identifying compounds that disrupt normal NHP interactions; or may themselves disrupt or activate such interactions.

The principle of the assays used to identify compounds that bind to NHP, or NHP ligands/accessory molecules, involves preparing a reaction mixture of NHP and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The NHP species used can vary depending upon the goal of the screening assay. For example, where agonists of the natural

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NHP accessory molecule or ligand are desired, full length NHP, or a soluble truncated NHP, a NHP peptide, or NHP fusion protein containing one or more NHP domains fused to a protein or polypeptide that affords advantages in the assay system (e.g., labeling, isolation of the resulting complex, etc.) can be utilized. Where compounds that directly interact with the NHP are sought, peptides corresponding to NHP and fusion proteins containing NHP, or a portion thereof, can be used.

The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the NHP, polypeptide, peptide, or fusion protein therefrom, or the test substance onto a solid phase and detecting NHP/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the NHP reactant may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously nonimmobilized component is pre-labeled, the

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detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for a NHP, polypeptide, peptide or fusion protein, or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Alternatively, cell-based assays can be used to identify compounds that interact with a NHP. To this end, cell lines that express a NHP, or cell lines (e.g., COS cells, CHO cells, fibroblasts, etc.) that have been genetically engineered to express a NHP or NHP ligand/accessory molecules (e.g., by transfection or transduction of NHP DNA, etc.) can be used. Interaction of the test compound with, for example, NHP ligand expressed by the host cell can be determined by comparison or competition with native NHP.

#### 5.5.2. ASSAYS FOR COMPOUNDS THAT INTERFERE WITH NHP RECEPTOR/INTRACELLULAR OR NHP/TRANSMEMBRANE MACROMOLECULE INTERACTION

Macromolecules that interact with NHP are referred to, for purposes of this discussion, as "binding partners". These binding partners are likely to be involved in NHP mediated biological pathways. Therefore, it is desirable to identify compounds that interfere with or disrupt the interaction of such binding partners which may be useful in regulating or augmenting NHP activity in the body and/or controlling

disorders associated with NHP activity (or a deficiency thereof).

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The basic principle of the assay systems used to identify compounds that interfere with the interaction between NHP, or  
5 NHP polypeptides, peptides or fusion proteins as described in  
Section 5.5.1 above (collectively, the NHP moiety), and its  
binding partner or partners involves preparing a reaction  
mixture containing the NHP moiety and the binding partner under  
conditions and for a time sufficient to allow the two to  
10 interact and bind, thus forming a complex. In order to test a  
compound for inhibitory activity, the reaction mixture is  
prepared in the presence and absence of the test compound. The  
test compound may be initially included in the reaction  
mixture, or may be added at a time subsequent to the addition  
15 of the NHP moiety and its binding partner. Control reaction  
mixtures are incubated without the test compound or with a  
placebo. The formation of any complexes between the NHP moiety  
and the binding partner is then detected. The formation of a  
complex in the control reaction, but not in the reaction  
20 mixture containing the test compound, indicates that the  
compound interferes with the interaction of the NHP moiety and  
the interactive binding partner. Additionally, complex  
formation within reaction mixtures containing the test compound  
and normal NHP can also be compared to complex formation within  
25 reaction mixtures containing the test compound and a mutant  
NHP. This comparison may be important in those cases wherein  
it is desirable to identify compounds that specifically disrupt  
interactions of mutant, or mutated, NHPs but not normal NHPs.

The assay for compounds that interfere with the  
30 interaction of the NHP moiety and its binding partners can be  
conducted in a heterogeneous or homogeneous format.  
Heterogeneous assays involve anchoring either the NHP moiety or  
the binding partner onto a solid phase and detecting complexes  
anchored on the solid phase at the end of the reaction. In



homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that  
5 interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance; *i.e.*, by adding the test substance to the reaction mixture prior to, or simultaneously with, the NHP moiety and interactive binding partner. Alternatively, test compounds  
10 that disrupt preformed complexes, *e.g.* compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

15 In a heterogeneous assay system, either the NHP moiety or an interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized  
20 by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the NHP moiety or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the  
25 species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete,  
30 unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on

the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

10       Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any  
15       complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

20       In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the NHP moiety and an interactive binding partner is prepared in which either the NHP moiety or its binding partners is labeled, but the signal generated by the label is quenched due  
25       to formation of the complex (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above  
30       background. In this way, test substances which disrupt NHP/intracellular binding partner interaction can be identified.

In a particular embodiment, a NHP fusion can be prepared for immobilization. For example, NHP or a peptide fragment can

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be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner can be purified and used to

5 raise a monoclonal antibody, using methods routinely practiced in the art and/or described above. This antibody can be labeled with the radioactive isotope  $^{125}\text{I}$ , for example, by methods routinely practiced in the art. In a heterogeneous assay, e.g., the GST-NHP fusion protein can be anchored to

10 glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the

15 system and allowed to bind to the complexed components. The interaction between the NHP moiety and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by

20 the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-NHP moiety fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test

25 compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the NHP moiety/binding partner interaction can be detected by adding the labeled

30 antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domain(s) of the NHP moiety and/or

the interactive or binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the binding sites.

5 These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensatory mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the

10 genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described above, and allowed to interact with and bind to its labeled binding

15 partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a relatively short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the

20 intracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, the NHP moiety

25 can be anchored to a solid material as described, above, by making a GST-NHP moiety fusion protein and allowing it to bind to glutathione agarose beads. The interactive binding partner can be labeled with a radioactive isotope, such as <sup>35</sup>S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage

30 products can then be added to the anchored GST-NHP moiety fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the intracellular binding partner binding domain, can be eluted, purified, and analyzed for amino acid sequence by well-known

